

# Aromatic Residues in the C-terminal Domain 2 Are Required for Nanog to Mediate LIF-independent Self-renewal of Mouse Embryonic Stem Cells\*

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Nanog was identified by its ability to sustain the LIF-independent self-renewal of mouse embryonic stem (ES) cells and has recently been shown to play a role in reprogramming adult fibroblasts into pluripotent stem cells. However, little is known about the structural basis of these remarkable activities of Nanog. We have previously identified an unusually strong transactivator named CD2 at its C terminus. Here we demonstrate that CD2 is required for Nanog to mediate ES cell self-renewal. Furthermore, deletion and point mutation analysis revealed that CD2 relies on at least seven aromatic amino acid residues to generate its potent transactivating activity. A mutant Nanog bearing alanine substitutions for these seven residues fails to confer LIF-independent self-renewal in mouse ES cells. Substitution of CD2 by the viral transactivator VP16 gave rise to Nanog-VP16, which is 10 times more active than wild-type Nanog in ES cells. Surprisingly, the expression of Nanog-VP16 in mouse ES cells induces differentiation and is thus unable to sustain LIF-independent self-renewal for mouse ES cells. Taken together, our results demonstrate that the CD2 domain of Nanog is a unique transactivator that utilizes aromatic residues to confer specific activity absolutely required for ES self-renewal.

Embryonic stem cells are the only pluripotent cells capable of generating the 200 or so cell types in our body and thus possess unmatched potentials to restore diseased or aged tissues or organs through transplantations (1, 2). As such, stem cell-based therapies are promising solutions to many of current unmet medical needs such as diabetes and Parkinson disease. Yet formidable obstacles have to be overcome before human ES<sup>2</sup> cells can be applied in human diseases both safely and efficaciously. Investigations into the basic biology of ES cells may provide

rational approaches to obstacles such as maintaining ES cells at the pluripotent state and inducing them to differentiate toward a specified lineage under culture conditions.

The pluripotency of mouse ES cells appears to be governed by a network of transcription factors including Oct4, Sox2, FoxD3, and Nanog (1, 3–6). Although volumes of data have been generated through large scale biological tools such as microarrays and proteomics about these core regulators of stem cell pluripotency (4, 7–9), little is known about the molecular mechanisms that govern their mechanism of action. To this end, we have focused on the transcription mechanism specified by Nanog, a gene known to sustain mouse ES cell self-renewal in the absence of LIF in culture conditions. We have defined two potent transactivators at its C terminus (2, 10, 11). One of them is CD2 (C-terminal domain 2), which has been shown to be as potent as the virally encoded VP16 in a Gal4-based transactivation assay (10). Here we demonstrate that CD2 is required for Nanog-mediated self-renewal of ES cells without LIF. Furthermore, we have uncovered an array of aromatic residues within CD2 that are required both for its transactivation activity and ES cell self-renewal.

## MATERIALS AND METHODS

**Cell Lines and Plasmids**—HEK 293T cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (Hyclone, UT) and antibiotics (penicillin and streptomycin, 100  $\mu$ g/ml) as described (10). P19 cells and F9 cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 15% fetal bovine serum (Hyclone, UT) and antibiotics (penicillin and streptomycin, 100  $\mu$ g/ml) (3, 10). Mouse ES cells (CGR8 ES) were cultured on 0.1% gelatin-coated substrates and cultured in Glasgow minimum essential medium (Sigma) supplemented with 20% fetal bovine serum (Invitrogen), 100 mM nonessential amino acids (Invitrogen), 0.55 mM mercaptoethanol (Sigma), 2 mM L-glutamine (Invitrogen), and 1,000 units/ml human recombinant LIF (Chemicon) as described (3, 5, 6, 10).

The expression plasmid pCR3.1-Gal4DBD was prepared as described (11). Gal4-CD2 and a series of truncated Gal4-CD2 plasmids were constructed by inserting a PCR fragment, which was amplified by reverse transcription-PCR from plasmids pCR3.1-NanogF (described in Ref. 11), to the downstream EcoRV site of pCR3.1-Gal4DBD as described (11). The primers

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<sup>2</sup> The abbreviations used are: ES, embryonic stem; RT, reverse transcription.

**TABLE 1**
**Primer for series of truncated Gal4-CD2 construction**

Name	Forward sequence (5'-3')	Reverse sequence (5'-3')
CD2	aatgctgctccgctccataac	tcatatttcacctgggtggag
CD2( $\Delta$ 7)	aatgctgctccgctccataac	tcaagagtagttcaggaataattcca
CD2( $\Delta$ 10)	aatgctgctccgctccataac	caggaataattccaaggcttggtg
CD2( $\Delta$ 13)	aatgctgctccgctccataac	tcatatttcacctgggtggag
CD2( $\Delta$ 20)	aatgctgctccgctccataac	aaaatgcatggtcttccctag
( $\Delta$ 6)CD2	aacttcggggaggacttttc	tcatatttcacctgggtggag
( $\Delta$ 8)CD2	ggggaggactttctgcagcc	tcatatttcacctgggtggag
( $\Delta$ 10)CD2	gactttctgcagccttacgt	tcatatttcacctgggtggag
( $\Delta$ 14)CD2	ccttacgtacagttgcagca	tcatatttcacctgggtggag
( $\Delta$ 16)CD2	gtacagttgcagcaaaactt	tcatatttcacctgggtggag
( $\Delta$ 20)CD2	caaaacttctctgccagtga	tcatatttcacctgggtggag

**TABLE 2**
**Primer for site-directed mutagenesis**

Name	Forward sequence (5'-3')	Reverse sequence (5'-3')
Gal4-LFL <sub>50-52</sub> mu	gccttggaagcagcgcgaactactctgtgactccacc	Ggtggagtcacagagtagttcgcggctgcttccaaggc
Gal4-NYS <sub>53-55</sub> mu	ccccacaagccttggaattattcctggcgcgcgtgtgac	gtcacagcggcggccaggaataattccaaggcttggtggg
Gal4-LFLNYS <sub>50-55</sub> mu	ccccacaagccttggaagcagcgcgcgcgcgtgtgac	gtcacagcggcggcgcgcgcgtgcttccaaggcttggtggg
Gal4-L <sub>50</sub> mu	ccccacaagccttggaagcattcctgaactactctgtgac	gtcacagagtagttcaggaatgcttccaaggcttggtggg
Gal4-L <sub>52</sub> mu	ccccacaagccttggaattattcgcgaactactctgtgactccaccaggt	Acctgggtggagtcacagagtagttcgcgaataattccaaggc
Gal4-LFL <sub>50-52</sub> mu	ccccacaagccttggaagcattcgcgaactactctgtgac	ttgtgggg
Gal4-F <sub>51</sub> mu	ccccacaagccttggaattagcctgaactactctgtgac	gtcacagagtagttcgcgaatgcttccaaggcttggtggg
Gal4-CD2-F/W mu	gccttggaattatggctgaactactctgtgac	gtcacagagtagttcagggttaattccaaggcttggtggg
Gal4-CD2-F/Y mu	gccttggaattatagcctgaactactctgtgactccacc	Gtcacagagtagttcagccataattccaaggc
Gal4-CD2-F/D mu	gccttggaattatagcctgaactactctgtgactccacc	Ggtggagtcacagagtagttcagggtataattccaaggc
Gal4-CD2-F/E mu	gccttggaattatagcctgaactactctgtgactccacc	Ggtggagtcacagagtagttcagggtataattccaaggc
Gal4-CD2-F/G mu	gccttggaattatagcctgaactactctgtgactccacc	Ggtggagtcacagagtagttcagggtataattccaaggc
Gal4-CD2-F/L mu	gccttggaattatagcctgaactactctgtgactccacc	Ggtggagtcacagagtagttcagggtataattccaaggc
Gal4-CD2-F/S mu	gccttggaattatagcctgaactactctgtgactccacc	Ggtggagtcacagagtagttcagggtataattccaaggc
Gal4-CD2-F/T mu	gccttggaattatagcctgaactactctgtgactccacc	Ggtggagtcacagagtagttcagggtataattccaaggc
Gal4-CD2-F/P mu	gccttggaattatagcctgaactactctgtgactccacc	Ggtggagtcacagagtagttcagggtataattccaaggc
Gal4-CD2-F/H mu	gccttggaattatagcctgaactactctgtgactccacc	Ggtggagtcacagagtagttcagggtataattccaaggc
Gal4-CD2-F/Q mu	gccttggaattatagcctgaactactctgtgactccacc	Ggtggagtcacagagtagttcagggtataattccaaggc
Gal4-CD2-F/R mu	gccttggaattatagcctgaactactctgtgactccacc	Ggtggagtcacagagtagttcagggtataattccaaggc
Gal4-CD2-F/I mu	gccttggaattatagcctgaactactctgtgactccacc	Ggtggagtcacagagtagttcagggtataattccaaggc
Gal4-CD2-F/M mu	gccttggaattatagcctgaactactctgtgactccacc	Ggtggagtcacagagtagttcagggtataattccaaggc
Gal4-CD2-F/N mu	gccttggaattatagcctgaactactctgtgactccacc	Ggtggagtcacagagtagttcagggtataattccaaggc
Gal4-CD2-F/T mu	gccttggaattatagcctgaactactctgtgactccacc	Ggtggagtcacagagtagttcagggtataattccaaggc
Gal4-CD2-F/K mu	gccttggaattatagcctgaactactctgtgactccacc	Ggtggagtcacagagtagttcagggtataattccaaggc
Gal4-CD2-F/V mu	gccttggaattatagcctgaactactctgtgactccacc	Ggtggagtcacagagtagttcagggtataattccaaggc
Gal4-CD2-F <sub>8</sub> mu	cgctccataacgcggggaggactttctgc	Gcagaaagtcctccccggcgttatggagcgg
Gal4-CD2-F <sub>12</sub> mu	ggggaggagcgtctgcagccttacgtacagtgtgc	Gcaactgtacgttaaggctgcagagcgtcctcccc
Gal4-CD2-F <sub>16</sub> mu	ctgcagcctgcgttacagttgcagcaaaacttctctgcc	Ggcagagaagttttgtgcaactgtacggcaggctgcag
Gal4-CD2-F <sub>12</sub> Y <sub>16</sub> mu	cgctccataacgcggggaggagcgtctgcagcctgcgc	cggcaggctgcagagcgtcctccccggcgttatggagcgg
Gal4-CD2-F <sub>23</sub> mu	cagttgcagcaaaacgcctctgccagtgatttggagg	Cctccaaatcactggcagagggcgttttgcgtgcaactg
Gal4-CD2-CD2-F <sub>42</sub> mu	gggaaagccatgcgcattgctagcaccaccaagcc	Ggcttgtgggggtgctagcagcgcagcgttctccc
Gal4-CD2-F <sub>54</sub> (W)mu	ccataactggggggaggagctggtgcagccttgggtacag	ctgtaccaaggcgtgcagcagcgtcctccccagttatgg
Gal4-CD2-F <sub>8</sub> F <sub>12</sub> Y <sub>16</sub> (W)mu	ggaattagcctgaacgcctctggtactccaccagg	Cctgggtggagtcacagagggcgttccagggcgaattcc
Gal4-CD2-F <sub>23</sub> (W)mu	cagttgcagcaaaactggtctggtcagtgatttggagg	Cctccaaatcactggcagcagcagtttctgctgcaactg
Gal4-CD2-F <sub>42</sub> (W)mu	gggaaagccatgcgcattggtgagcaccaccaagcc	Ggcttgtgggggtgctccaatgctgagcgttctccc

used as shown in Table 1. A series of mutant Gal4-CD2 were generated by site-directed mutagenesis (described in Ref. 11); the primers used are shown in Table 2.

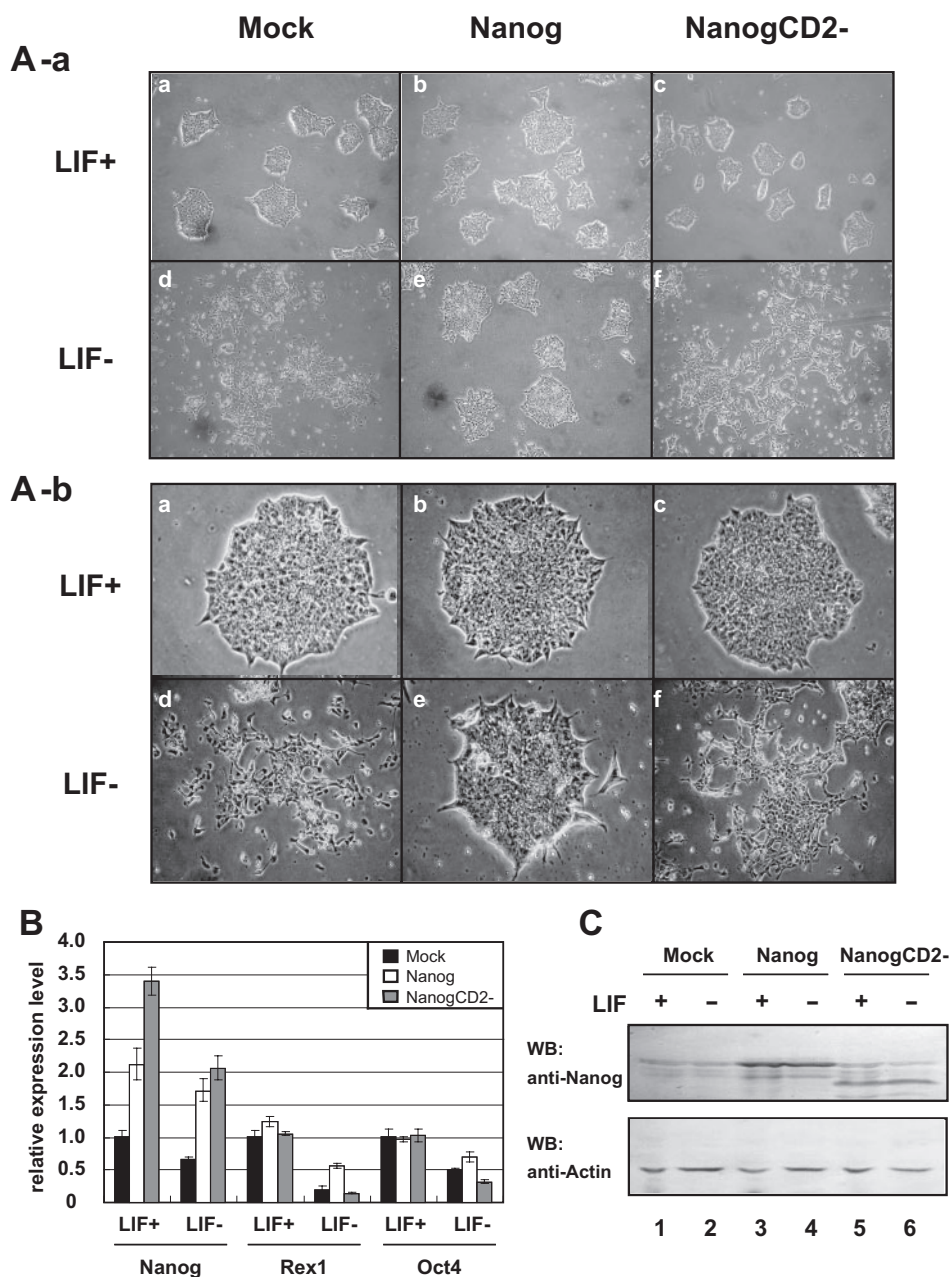
The plasmids used for generating ES stable lines were constructed by inserting a PCR fragment, which was amplified by reverse transcription-PCR from plasmids pCR3.1-Nanog, pCR3.1-Nanog-mu1, and pCR3.1-Nanog-mu2 (generated through site-directed mutagenesis with above primers in pCR3.1-Nanog as template), to the MCS xho1 and NotI site of pPyCAGIP vector. The primers used are as follows: forward, 5'-atactcgagaccatgagtg-gggtctcc-3'; reverse, 5'-tatgcccgcctcatatttcacctggtggag-3'.

**Transfections, Western Blotting, and Reporter Assay**—HEK293T cells were transfected by calcium phosphate co-precipitation methods. F9, P19, and CGR8 ES cells were transfected by Lipofectamine 2000 (Invitrogen). For Western blotting analysis, HEK293T cells cultured in 24-well tissue culture plates were transfected by a calcium phosphate

**TABLE 3**
**Primer for real time PCR**

Name	Forward sequence (5'-3')	Reverse sequence (5'-3')
Actin	agtgtgacgttgacatccgt	Tgctaggagccagagcagta
Nanog	ctcaagtcctgaggtgaca	Tgaacctgtccttgagtc
Rex-1	cagccagaccaccatctgtc	Gtctccgatttgcatactctctg
Oct4	aggccagtcacgaataaccag	taggtatccgtcagggagac

co-precipitation method with expression plasmids (0.8  $\mu$ g each) as described (11). After transfection (24 h), the cells were washed by phosphate-buffered saline and lysed on ice by radioimmune precipitation assay buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.25% sodium deoxycholate, 0.1% Nonidet P-40, 0.1% Triton X-100) for 10 min and cleared of debris by centrifugation at 15,000 rpm for 15 min at 4 °C. After boiling with an equal volume of 2 $\times$  SDS loading buffer for 5 min, the cell lysates were electrophoresed with 10% SDS-PAGE and blotted to polyvinylidene difluoride membranes (Mil-



**FIGURE 1. ES cells expressing CD2-deficient Nanog fail to undergo self-renewal in the absence of LIF.** A, morphology of CGR8 ES cells with constitutive expression of vector (mock), wild-type Nanog, and Nanog CD2-mutant, cultured with or without LIF for 5 days. The pictures in panels 1 and 2 were taken with different magnification, respectively, as indicated in each panel. B, expression of the pluripotency marker, including Nanog, Oct4, and Rex1 of above cells by quantitative RT-PCR. The cells were cultured in as A for 5 days, and RNAs were isolated from these cells and assayed by quantitative RT-PCR. All of these data were derived from triplet PCRs and had been repeated three times and calculated with  $\beta$ -actin as internal reference as labeled on the figure. C, Western blotting analysis of Nanog and NanogCD2 in stable cell lines. The cells described in A were extracted with radioimmune precipitation assay buffer and analyzed by Western blot analysis with anti-Nanog antibody and anti-actin antibody (as internal reference), respectively. All of the proteins were at the expected size both with LIF and without LIF.

lipore). The membranes were then blotted with 5% nonfat milk and incubated with anti-FLAG antibody (1:5000), followed by alkaline phosphatase-conjugated anti-mouse (1:5000) second antibodies. The membranes were then washed extensively and developed by incubating in a solution containing nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate.

For reporter assays, the cells seeded in 24-well plates were transiently transfected with p5G-e1b-luciferase (0.2  $\mu$ g/well) and

effector plasmids (0.4  $\mu$ g/well) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instruction. pCMV-*Renilla* plasmids (0.002  $\mu$ g/transfection; Promega, WI) were co-transfected in each well as internal references, and the DNA concentrations for all transfections were normalized to equal amounts with the parental pCR3.1 vector. 36 h later, the cells were washed by phosphate-buffered saline and lysed by 50  $\mu$ l of 1 $\times$  PLB buffer (Promega, WI). Luciferase activity was measured using a dual luciferase reporter assay system (Promega) and a TD2020 Luminometer (Turner Design). Each transfection was carried out in duplicate and repeated at least five times.

**Real Time Reverse Transcription (RT)-PCR Analysis**—2  $\mu$ g of total RNA was reverse transcribed in a final volume of 20  $\mu$ l as previously described (3). PCRs were undertaken using the real time PCR Master Mix (SYBR GREEN) reagent kit (Toyobo), according to the manufacturer's protocol. PCR was performed in 15  $\mu$ l of total volume for 45 cycles. The primers used are shown in Table 3.

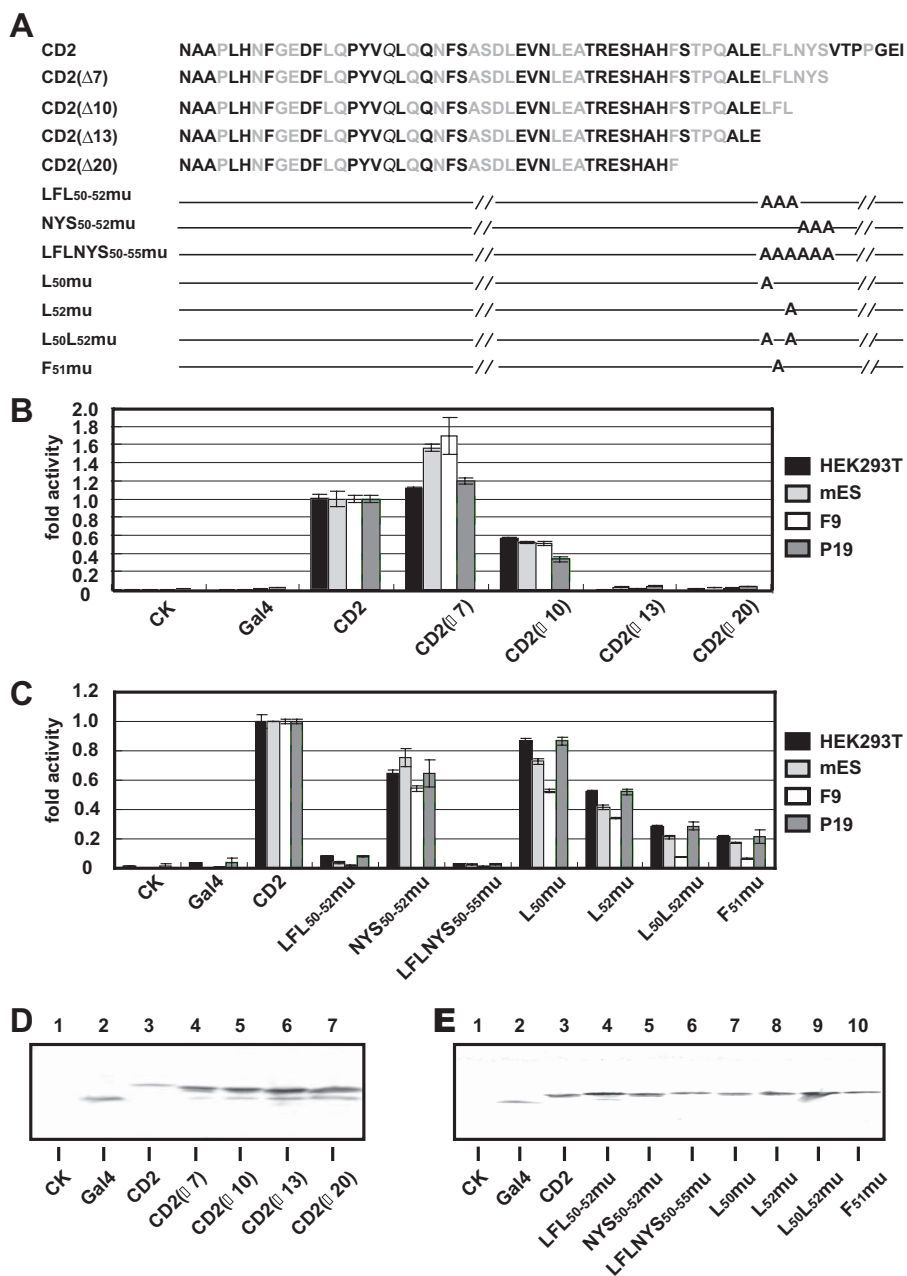
**Stable Cell Line Selection**—Feeder free ES cells (CGR8) maintained in ES medium containing 10<sup>3</sup>u/ml LIF (Chemicon) were seeded in 3.5-cm dishes and transfected with 2  $\mu$ g of each expression plasmid. 24 h after transfection, the cells were divided by 1:50 and seeded to new 6-cm dishes for selection. Puromycin (2  $\mu$ g/ml; Invitrogen) was added to the medium for selection. After selection for 10 days, single clone was picked up and expanded in 12-well tissue culture plate for further Western and real time RT-PCR analysis.

## RESULTS

### *The CD2 of Nanog Is Required for Nanog-mediated LIF-independent ES Cell Self-renewal*

Nanog was discovered based on its ability to sustain LIF-independent ES cell self-renewal, presumably by repressing the expression of genes involved in specifying the primitive endoderm lineage (5, 6). Although the repressor function of Nanog remains to be demonstrated, we have demonstrated that Nanog encodes two potent transactivators, WR and CD2 (10, 11). Because CD2 is more potent than WR in mediating transcription using the Gal4 system (10), we wished to assess the role of CD2 in medi-





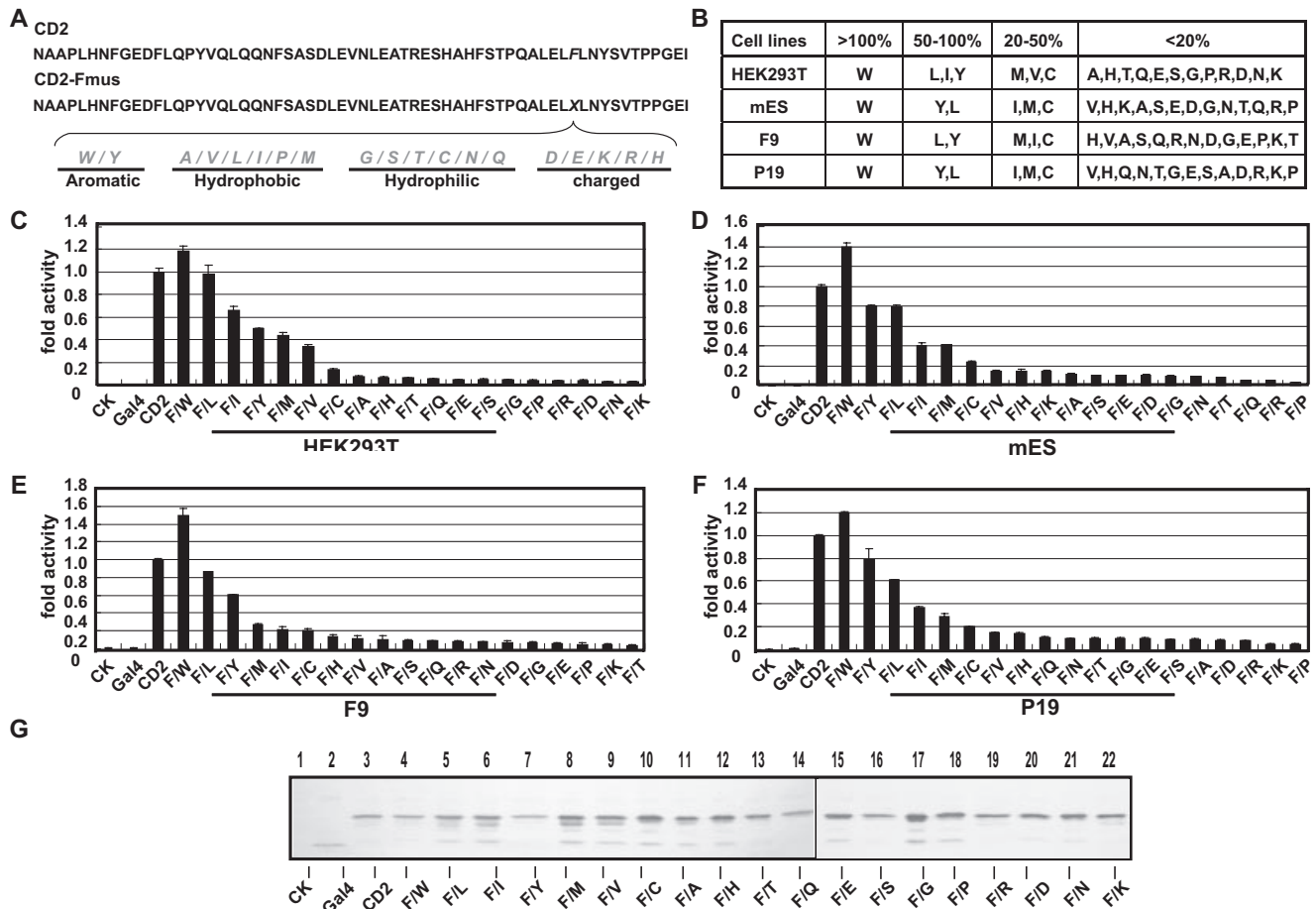
**FIGURE 2. Identification of critical residues at the C terminus of CD2 for transactivation activity.** *A*, schematic illustration of Nanog CD2 and deletions and point mutations. *B* and *C*, the activities of CD2 and its mutants shown in *A* as assayed in the Gal4-reporter system. Control and mutant plasmids were co-transfected with the reporter and *Renilla* reference vectors into HEK293T, mES, F9, and P19 cells as described under “Materials and Methods.” The luciferase activities of each transfections were assayed 24 h post-transfection using dual reporter assay systems (Promega). The results were the average of two independent experiments, and the error bars were derived from standard deviations. *D* and *E*, Western blot analysis of plasmids depicted in *A* as expressed in HEK293T cells. The cell lysates were processed and probed with anti-FLAG antibody as described (11). These constructs express proteins of the expected sizes.

ating LIF-independent ES cell self-renewal. We have previously generated a CD2-truncated Nanog and demonstrated that it remains active in mediating the transactivation of reporters bearing Nanog-binding sites (10). To begin to assess the role CD2 in ES cell self-renewal, this mutant was cloned into the expression vector pPyCAGIP (a gift from Chambers (6)) and stably transfected into mouse ES cells. As shown in Fig. 1A (panel 1), mouse ES cells transfected with control vector (panels *a* and *d*) underwent spontaneous differentiation in the

absence of LIF (panel *d* versus panel *a*) morphologically, forming disorganized clumps of flat cells. As expected, ES cells overexpressing wild type Nanog showed pluripotent morphology in the absence of LIF (Fig. 1A, panels *e* versus panels *b*) with clones of small and compact cells (6). On the other hand, ES cells expressing CD2-truncated Nanog became differentiated in the absence of LIF as the control (Fig. 1A, panels *f* versus panels *d*), suggesting that the CD2 domain is required for Nanog-mediated LIF-independent ES cell self-renewal. Representative clones from similar experiments in Fig. 1A (panel 1) were shown with higher magnification in Fig. 1A (panel 2). To further confirm the pluripotent states of the cells in Fig. 1A, we analyzed the expression levels of pluripotent markers Nanog, Oct4, and Rex1 by quantitative RT-PCR as presented in Fig. 1B. In the absence of LIF, ES cells expressing the CD2 truncation mutant did not sustain the expression of both Rex1 and Oct4, whereas ES cells expressing the wild type Nanog did (Fig. 1B). Because the primers we used for Nanog detection were designed for both endogenous and the transfected Nanog constructs in Fig. 1B (left column), we estimate that the contribution of exogenous Nanog or NanogCD2 to the overall Nanog expression levels to be  $\sim 1\times$  or  $2\times$ , respectively, in agreement with the results from Western blots shown in Fig. 1C. Together, these results demonstrate that CD2 is critical for Nanog-mediated LIF-independent ES cell self-renewal.

*Aromatic Amino Acids Are Critical for the Transactivation Function of CD2*—We then wished to determine the structural requirement for

CD2 transactivation activity. To discern the critical subdomain or residues, we made serial deletions of CD2 to generate CD2( $\Delta$ 7), CD2( $\Delta$ 10), CD2( $\Delta$ 13), and CD2( $\Delta$ 20) in fusion forms to the DNA-binding domain of Gal4, respectively (Fig. 2A). These constructs were then evaluated with a reporter construct, p5G-elb-luciferase as described (10, 11) (“Methods and Materials”), into HEK293T, mouse ES cells, F9 germ tumor cells, and P19 germ tumor cells, respectively, and their activities were quantified as described (11) and presented in



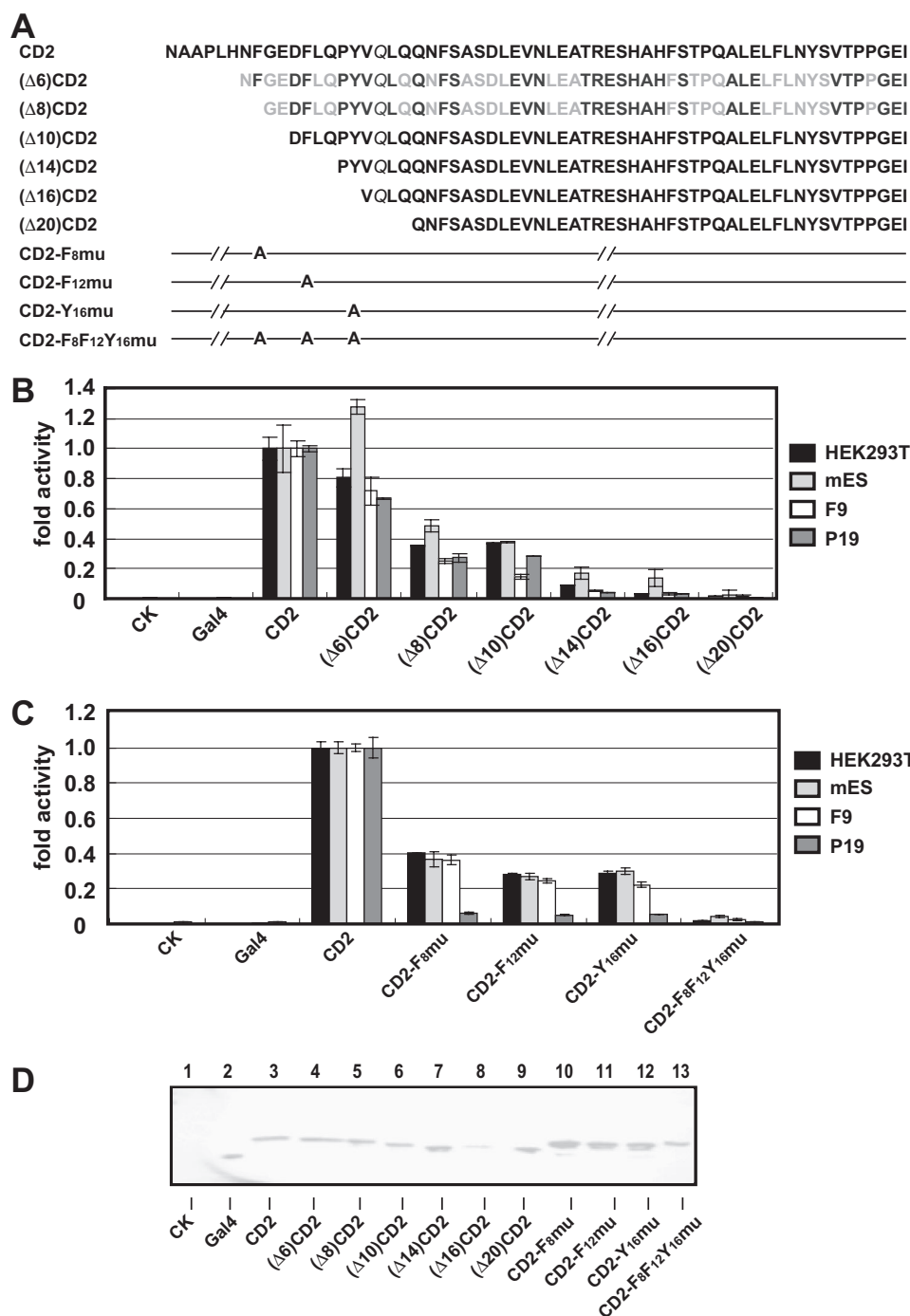
**FIGURE 3. Aromatic amino acids are preferred at Phe<sup>51</sup> for CD2 transactivity.** A, schematic illustration of CD2 and various substitutions at Phe<sup>51</sup> by other 19 amino acids. B, summary of the transactivation activity determined in C–F ranked by activity as >100%, 50–100%, 20–50%, and <20%. C–F, transcription activity of each protein from A as assayed in Fig. 2 (B and C). G, Western blot analysis of constructs from A in HEK293T cells. The cell lysates were processed and probed with anti-FLAG antibody as described (11).

Fig. 2B. These results suggest that the sequence <sup>50</sup>LFL may play a critical role in CD2 activity (CD2( $\Delta$ 13) versus CD2( $\Delta$ 10)), whereas the deletion of <sup>53</sup>NYS also reduces its activity significantly. We then performed alanine substitution individually or in combination for the <sup>50</sup>LFLNYS region as illustrated in Fig. 2A (lower panel). Their corresponding activities were measured as in Fig. 2B and presented in Fig. 2C. Consistent with the deletion data in Fig. 2B, substitutions at LFL resulted in almost complete loss of transactivation activity, whereas substitutions at NYS had about 40% reduction (Fig. 2C). Individual substitution at Leu<sup>50</sup>, Leu<sup>52</sup>, or Phe<sup>51</sup> led to progressive reduction of activity (Fig. 2C). Interestingly, F9 cells appeared to be more sensitive toward these substitutions (Fig. 2C). All of these constructs were well expressed at the protein level as demonstrated in Fig. 2 (D and E). Together, these results defined the critical role of <sup>50</sup>LFL, particularly Phe<sup>51</sup> in CD2 activity.

We then focused on Phe<sup>51</sup> and performed a systematic substitution to determine which amino acid residue is preferred at this position. We mutated Phe<sup>51</sup> into the rest of the amino acid family, including aromatic, hydrophobic, hydrophilic, and charged amino acids as shown in Fig. 3A. The activity of each substitution was determined in HEK293T,

mES, F9, and P19 cells as described in Fig. 2 and presented in Fig. 3 (C–F), and summarized in Fig. 3B. Interestingly, tryptophan, an aromatic residue, was able to replace Phe<sup>51</sup> completely, generating equal or more robust activity in all four cell lines tested. Substitution of Phe<sup>51</sup> with Leu, Ile, or Tyr resulted in CD2 with activities ranging from 50 to 100% of the wild type transactivator (Fig. 3B). Other substitutions led to more severe reduction of activity such as Phe  $\rightarrow$  Glu or Phe  $\rightarrow$  Lys (Fig. 3, B–F). Western blot analysis revealed similar levels of expression of these constructs (Fig. 3G). Based on these results, we conclude that the amino acid at Phe<sup>51</sup> should be an aromatic residue.

To define additional structural feature for CD2, we performed N-terminal deletions and alanine substitutions as illustrated in Fig. 4A. Transactivation activity data presented in Fig. 4B revealed a progressive loss of activity between  $\Delta$ 6 to  $\Delta$ 20. The deletion mutant ( $\Delta$ 20)CD2 lost almost all of its activity (Fig. 4B). Within the 20-amino acid segment deleted, there are two Phe residues and one Tyr residue that may contribute to the transactivating activity of CD2. To test this idea, we mutated all three of these residues to Ala as illustrated in Fig. 4A and measured the activity as presented in Fig. 4C. All three individual substitutions resulted in significant reduc-



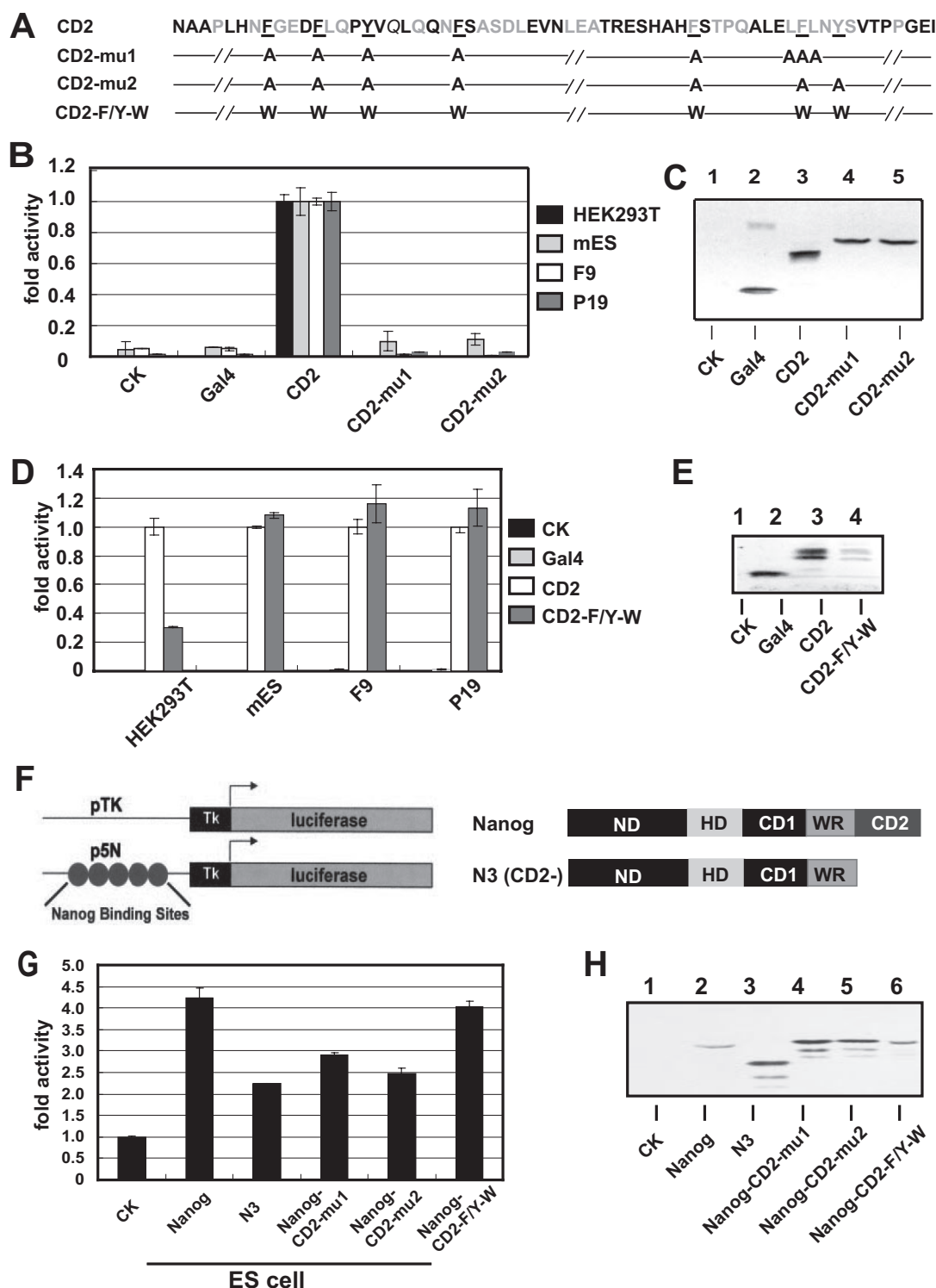
**FIGURE 4. Identification of critical residues at the N terminus of CD2 for its transcription activity.** *A*, schematic illustration of N-terminal mutations for CD2. *B* and *C*, transcription activity of each mutant depicted in *A* was assayed in four cell lines as described in Fig. 3. *D*, Western blot analysis of constructs from *A* expressed in HEK293T cells and probed with anti-FLAG antibody as described (11).

tion of CD2 activity, whereas the combined mutation CD2-F8F12Y16Mu lost its activity entirely (Fig. 4C), suggesting that these three aromatic residues play a critical role in maintaining CD2 activity.

**Requirement of Aromatic Residues in CD2 for LIF-independent ES Cell Self-renewal**—Aromatic residues have been demonstrated to play a critical role in mediating the transactivation function of the potent transactivator VP16 (12). Our mutagenesis studies presented above also indicate that the aromatic res-

idues within CD2 are of critical importance in mediating its transactivation function. As shown in Fig. 1, CD2 is required for Nanog to mediate ES cell self-renewal. So, we tested the role of these aromatic residues in Nanog-mediated ES cell self-renewal. First, we designed three CD2 mutants carrying multiple substitutions as shown in Fig. 5A, CD2-mu1 with alanine substitutions at positions Phe<sup>8</sup>-Phe<sup>12</sup>-Tyr<sup>16</sup>, Phe<sup>23</sup>-Phe<sup>42</sup>, and Leu<sup>50</sup>-Phe<sup>51</sup>-Leu<sup>52</sup>; CD2-mu2 with alanine substitutions of the seven aromatic amino acids at positions Phe<sup>8</sup>, Phe<sup>12</sup>, Tyr<sup>16</sup>, Phe<sup>23</sup>, Phe<sup>42</sup>, Phe<sup>51</sup>, and Tyr<sup>54</sup>; and CD2-F/Y-W with tryptophan substitutions of the seven aromatic amino acids at the same positions as CD2-mu2. Both alanine substitution mutants, CD2-mu1 and CD2-mu2, are inactive in the Gal4 reporter assay system as shown in Fig. 5B in all four different cell types. However, the tryptophan substitution mutant CD2-F/Y-W remains almost equal activity as wild type CD2 in all three pluripotent cell types (F9, P19, and mES), but with less activity in HEK293T as shown in Fig. 5D. We then engineered all three mutants back into the full-length Nanog and then inserted these fragments into both PCR3.1-FLAG and pPyCAGIP expression vectors as shown in Fig. 5F. Employing p5N, which harbors five copies of the Nanog-binding site, as a reporter (described in Fig. 5F) (10), we assayed the transactivation activity of these mutants in mES cells. Consistent with the results in Gal4 reporter system, N3, a Nanog mutant with CD2 truncated, is ~50%, Nanog-CD2-mu1 and Nanog-CD2-mu2 ~70%, and Nanog-CD2-F/Y-W ~100% as wild type Nanog (Fig. 5G). These con-

structs generated stable protein products as revealed by Western blot analysis (Fig. 5H). We then generated stable clones in ES cells. Representative clones from ES cell carrying control vector, Nanog, Nanog-CD2-mu1, Nanog-CD2-mu2, and Nanog-CD2-F/Y-W were grown in the absence or presence of LIF (Fig. 5I, panels 1 and 2) and then analyzed for Nanog expression by Western blotting (Fig. 5K). As shown in Fig. 5K, the transgenes were expressed in the ES cell clones, especially in the absence of LIF (lanes 4, 6, 8, and 10) as expected. We then



**FIGURE 5. Substitution of aromatic residues in CD2 by Ala, but not Trp, abolished the transactivation activity of CD2 and its self-renewal activity of Nanog.** *A*, schematic illustration of CD2 and its aromatic residue substitution mutants. *B* and *D*, transcription activity of CD2 and those mutants in assayed in the Gal4-reporter system in four cell lines as indicated. Note that CD2-mu1 and -mu2 lost all their activity, whereas CD2-F/Y-W remains fully active in mES, F9, and P19 cells. *C* and *E*, Western analysis of Gal4-CD2 and its mutants with anti-FLAG antibody as described (11). *F*, left panel, reporters for full-length Nanog. Right panel, Nanog and CD2 deficient N3. *G*, transcription activity of Nanog, N3, and Nanog-CD2-mu1, -mu2, and -F/Y-W determined with p5N reporter as described (11). The results were the average of two independent experiments with triplicates, and the error bars derived from standard deviations. *H*, Western blot analysis of constructs used in *G* and each construct expressed at the expected size. *I*, morphology of CGR8 ES cells constitutively expressing the vector (mock), wild-type Nanog, and Nanog-CD2-mu1, Nanog-CD2-mu2, or Nanog-CD2-F/Y-W cultured with or without LIF for 5 days. The pictures in I-1 and I-2 were taken with different magnifications. Note that CD2-F/Y-W behaves as the wild-type Nanog in promoting ES cell self-renewal. *J*, expression level of the pluripotency markers Nanog, Oct4, and Rex1. After being cultured both with or without LIF for 5 days, RNAs of above cells described in *I* were assayed by quantitative RT-PCR. The relative fold values were derived based on the values for mock ES cells cultured with LIF, which had all three values set as 1. *K*, cell lysates of cells described in *I* were isolated with radioimmune precipitation assay buffer and then analyzed by Western blot (WB) with anti-Nanog antibody and anti-actin antibody (as internal reference), respectively.

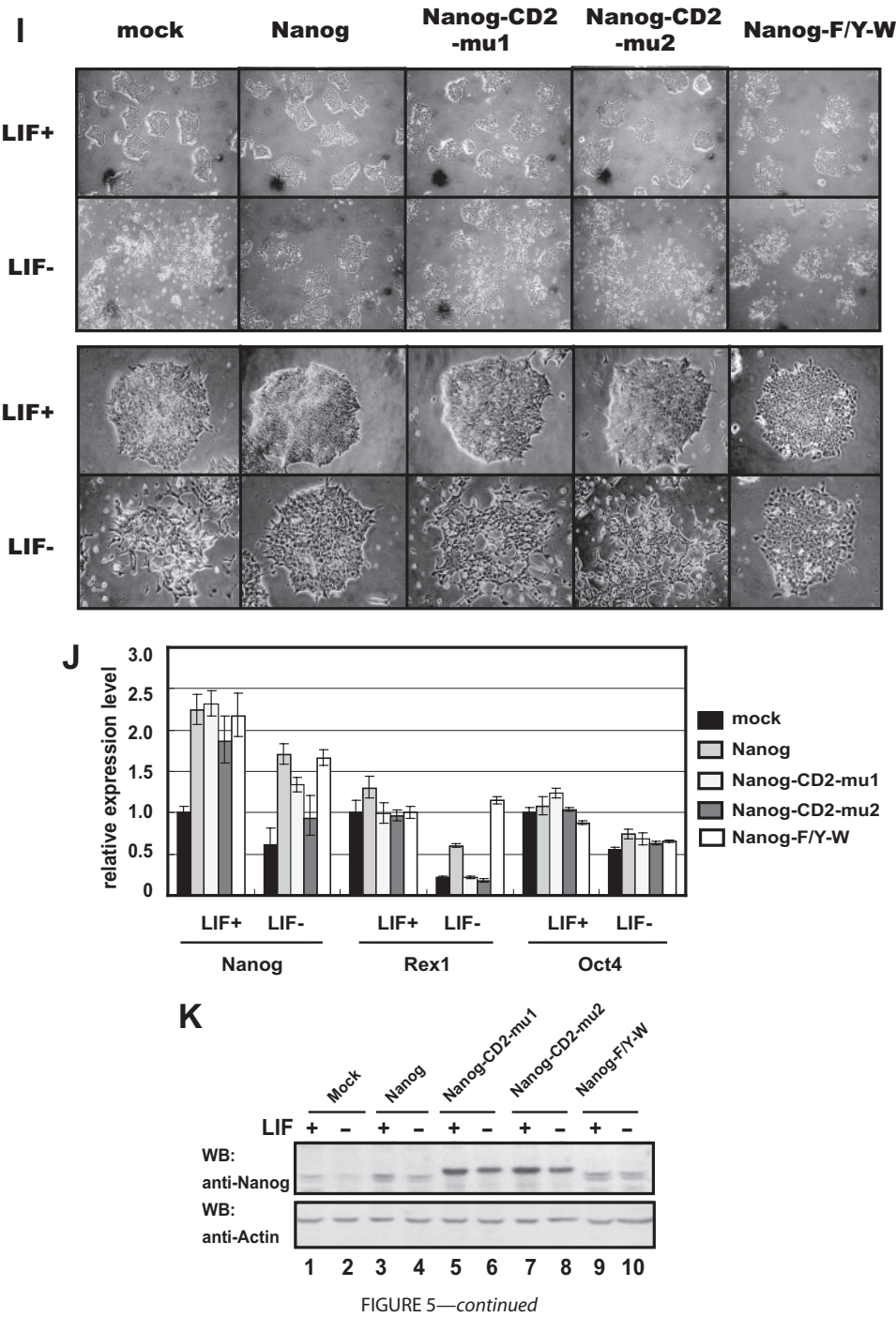


*The Self-renewing Activity of CD2 Cannot Be Substituted by VP16—*  
We have shown previously, based on the Gal4 reporter system, that CD2 is almost as active as VP16 (10, 11). To see whether VP16 is capable of substituting the activity of CD2 in both transactivation activity and pluripotency, we engineered Nanog-VP16 as shown in Fig. 6A. Employing the p5N reporter system, this mutant reveals a ~12-fold activity compared with wild type Nanog in mouse ES cells (shown in Fig. 6C). However, when we introduced the pPyCAGIP-Nanog-VP16 into mES cells as described in Fig. 1, it fails to sustain ES cell self-renewal and but induces ES cells differentiation morphologically (shown in Fig. 6B). Western blot analysis shown in Fig. 6D revealed that the transgene of Nanog-VP16 is well expressed (*upper band*), whereas the endogenous Nanog declined in expression even in the presence of LIF. Accordingly, the expression of differentiation markers were induced by Nanog-VP16 based on both RT-PCR (Fig. 6E) and quantitative RT-PCR (Fig. 6F) analyses. Surprisingly, Nanog-VP16 appears to have induced the ES cells differentiating into primitive endoderm, mesoderm, and even trophoblast based on these markers. These results suggest that CD2 plays an critical role in mediating Nanog-dependent ES cells self-renewal, which may not be substituted by other transactivation domain.

## DISCUSSION

Here we demonstrate that 1) the CD2, a strong transactivator at the C terminus of Nanog, is required for Nanog-mediated ES cell self-renewal; 2) the transactivation

activity of CD2 is dependent on aromatic residues; and 3) the self-renewing activity of CD2 cannot be substituted by VP16. These findings are significant for the following reasons: 1) Although Nanog is recognized as a key regulator of ES cell pluripotency and its overexpression alone can sustain ES cell self-renewal independent of LIF, the mechanism of action for Nanog remains undefined. We first reported the transactivation function of Nanog but did not establish the role of transactivation function of Nanog in stem cell self-renewal. In this report, we presented evidence that CD2 activity is required for Nanog mediated self-renewal of ES cells. Our results from Nanog-VP16 appear to suggest that CD2 func-



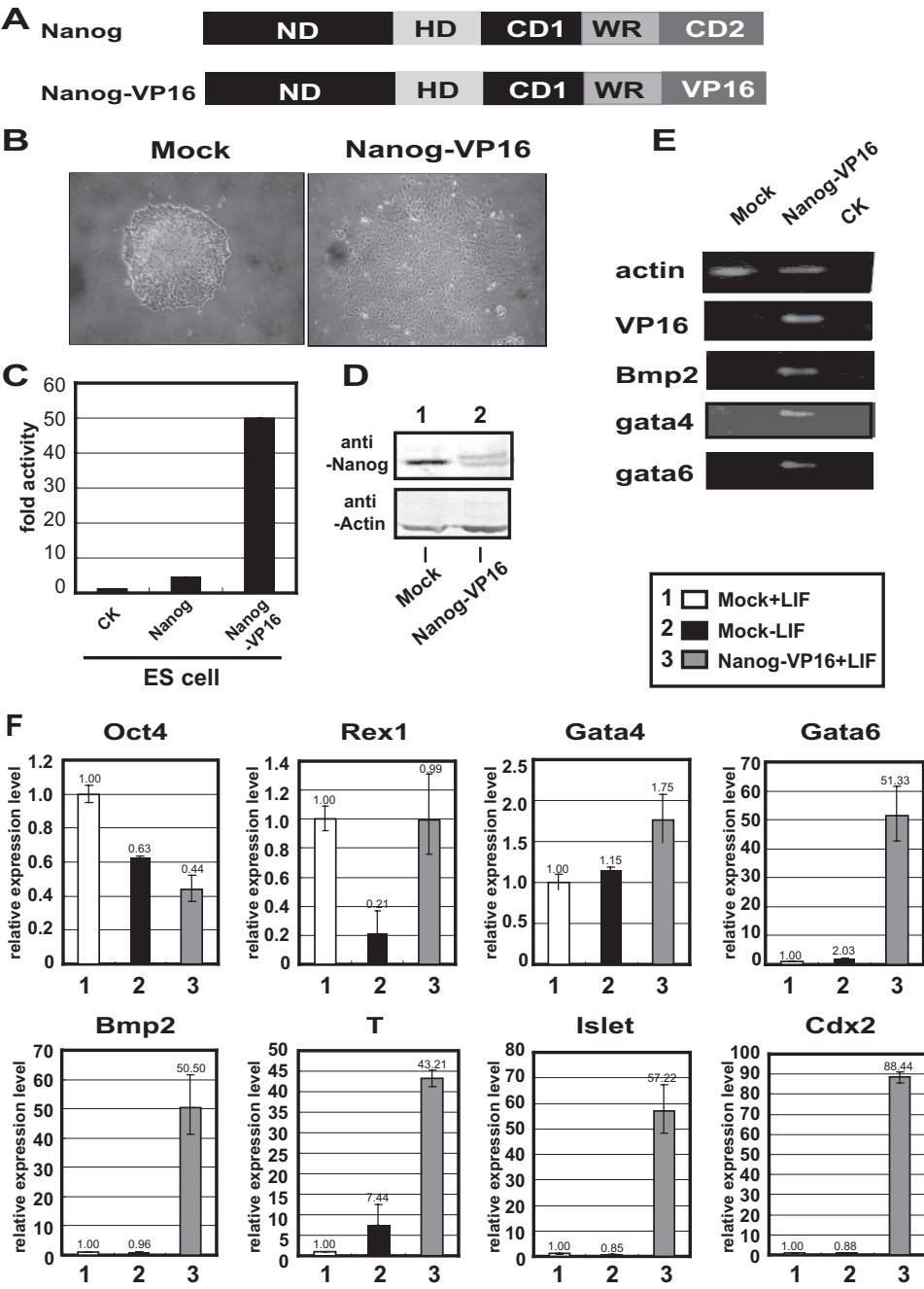
tested whether Nanog-CD2-mu1, -mu2, and -F/Y-W are able to sustain ES cell self-renewal in the absence of LIF. As shown in Fig. 5I, ES cells carrying the Nanog and Nanog-CD2-F/Y-W transgene remain pluripotent morphologically, whereas those carrying Nannog-CD2-mu1 and -mu2 failed to maintain their pluripotent morphology. The morphological phenotypes observed in Fig. 5I were corroborated by the expression of pluripotent markers such as Nanog, Oct4, and Rex-1 as measured by quantitative RT-PCR (Fig. 5J). Based on these observations, we conclude that Nanog relies on the aromatic residues in the CD2 transactivation domain to maintain ES cell self-renewal and pluripotency.



our previous reports, CD2 does not have any known structural features found in other transactivation domains and thus may regulate transcription through a distinct mechanism. Our approaches involving N- and C-terminal deletion analysis coupled with point mutagenesis revealed that aromatic residues play a critical role in sustaining CD2 activities. Interestingly, VP16, the most potent transactivator reported so far, utilizes aromatic residues to mediate strong transcription activity (12). Although CD2 and VP16 share little sequence homology, the similar requirement for aromatic residues suggests that they may share a common organizing principle based on aromatic residues. Structural determination of CD2 and VP16 may help to confirm this prediction. 3) By mutating the aromatic residues in CD2, we have generated a Nanog mutant that cannot sustain LIF-independent self-renewal yet does not interfere with the activity of endogenous Nanog. One would have expected that Nanog-CD2-mu1 or -mu2 behave as dominant negative mutants as we demonstrated for Oct4 and Sox2. ES cells expressing both mutants remain pluripotent in the presence of LIF, suggesting that endogenous Nanog functions well in the presence of these two mutants.

Our experimental approach exploits the unique property of Nanog in its ability to sustain ES cell self-renewal in the absence of LIF upon overexpression. By quantitative RT-PCR, we estimated that the wild type Nanog transgene is expressed at  $\sim 1\times$  the endogenous level, whereas the mutants at  $\sim 2\text{--}3\times$  the endogenous genes (Fig. 1B). Apparently, the level of expression contributed from the wild type Nanog

transgene is sufficient to maintain the pluripotency of ES cells in the absence of LIF for the duration of our assay. Interestingly, these ES cells can be induced to undergo differentiation in the presence of RA (data not shown), suggesting that the amount of Nanog contributed by the transgene in our experimental system is insufficient to prevent ES cell differentiation. This obser-



**FIGURE 6. VP16 fails to substitute CD2 for Nanog-mediated ES cell self-renewal.** A, schematic illustration of Nanog, and Nanog-VP16 mutant. B, morphology of ES cells expressing the vector (Mock) and Nanog-VP16 cultured with LIF. C, transcription activity of Nanog and Nanog-VP16. Nanog and Nanog-VP16 were inserted into pPyCAGIP vector and then transfected into mES cells as described under "Materials and Methods" (10). D, cell lysates of cells described in B were analyzed by Western blotting with anti-Nanog antibody and anti-actin antibody (as internal reference), respectively. E, RT-PCR analysis of various markers in mock and Nanog-VP16 ES cells cultured in LIF. Actin, VP16, Bmp2, Gata4, and Gata6 were analyzed as indicated. F, expression of pluripotency and differentiation markers in Nanog-VP16 cells. Oct4, Rex1, Gata4, Gata6, Bmp2, T, Islet, and Cdx2 representing pluripotent and differentiated states were analyzed in mock and Nanog-VP16 cells by quantitative RT-PCR. All of the relative fold values were derived based on values expressed in mock ES cells cultured with LIF set as 1.

tion in ES cell self-renewal is unique and cannot be replaced by other transactivation domains (Fig. 6). Thus, further delineation of downstream genes regulated by CD2 would greatly enhance our understanding of Nanog-mediated ES cell self-renewal. 2) We were surprised that the most critical residues within CD2 are aromatic residues. As pointed out in

our previous reports, CD2 does not have any known structural features found in other transactivation domains and thus may regulate transcription through a distinct mechanism. Our approaches involving N- and C-terminal deletion analysis coupled with point mutagenesis revealed that aromatic residues play a critical role in sustaining CD2 activities. Interestingly, VP16, the most potent transactivator reported so far, utilizes aromatic residues to mediate strong transcription activity (12). Although CD2 and VP16 share little sequence homology, the similar requirement for aromatic residues suggests that they may share a common organizing principle based on aromatic residues. Structural determination of CD2 and VP16 may help to confirm this prediction. 3) By mutating the aromatic residues in CD2, we have generated a Nanog mutant that cannot sustain LIF-independent self-renewal yet does not interfere with the activity of endogenous Nanog. One would have expected that Nanog-CD2-mu1 or -mu2 behave as dominant negative mutants as we demonstrated for Oct4 and Sox2. ES cells expressing both mutants remain pluripotent in the presence of LIF, suggesting that endogenous Nanog functions well in the presence of these two mutants.

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vation is in contrast to those originally reported by Chambers *et al.* (6) and Matsui *et al.* (5). This discrepancy may reflect the expression levels of the transgene delivered by the different expression systems (episomal *versus* integrated). The episome strategy employed by Chambers *et al.* and Matsui *et al.* in general can deliver much stronger expression than the integrated approach we adopted. Nevertheless, our results suggest that Nanog function can be analyzed in wild type ES cells upon over-expression at  $\sim 1\times$  that of endogenous level.

REFERENCES

1. Pan, G. J., Chang, Z. Y., Scholer, H. R., and Pei, D. (2002) *Cell Res.* **12**, 321–329
2. Pan, G., and Thomson, J. A. (2007) *Cell Res.* **17**, 42–49
3. Pan, G., Li, J., Zhou, Y., Zheng, H., and Pei, D. (2006) *FASEB J.* **20**, 1730–1732
4. Boyer, L. A., Lee, T. I., Cole, M. F., Johnstone, S. E., Levine, S. S., Zucker, J. P., Guenther, M. G., Kumar, R. M., Murray, H. L., Jenner, R. G., Gifford, D. K., Melton, D. A., Jaenisch, R., and Young, R. A. (2005) *Cell* **122**, 947–956
5. Mitsui, K., Tokuzawa, Y., Itoh, H., Segawa, K., Murakami, M., Takahashi, K., Maruyama, M., Maeda, M., and Yamanaka, S. (2003) *Cell* **113**, 631–642
6. Chambers, I., Colby, D., Robertson, M., Nichols, J., Lee, S., Tweedie, S., and Smith, A. (2003) *Cell* **113**, 643–655
7. Assou, S., Le Carrou, T., Tondeur, S., Strom, S., Gabelle, A., Marty, S., Nadal, L., Pantesco, V., Reme, T., Hugnot, J. P., Gasca, S., Hovatta, O., Hamamah, S., Klein, B., and De Vos, J. (2007) *Stem Cells* **25**, 961–973
8. Orkin, S. H. (2005) *Cell* **122**, 828–830
9. Wang, J., Rao, S., Chu, J., Shen, X., Levasseur, D. N., Theunissen, T. W., and Orkin, S. H. (2006) *Nature* **444**, 364–368
10. Pan, G., and Pei, D. (2005) *J. Biol. Chem.* **280**, 1401–1407
11. Pan, G. J., and Pei, D. Q. (2003) *Cell Res.* **13**, 499–502
12. Regier, J. L., Shen, F., and Triezenberg, S. J. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90**, 883–887





